

## **SPECIFIC MODULATION OF TH1/TH2 CYTOKINE EXPRESSION BY RIBAVIRIN IN ACTIVATED T-LYMPHOCYTES**

This application is a continuation-in-part of co-pending, allowed US Ser. No. 09/156,646, filed September 18, 1998, which is a continuation in part of US 09/097450, filed June 15, 1998, issued on May 16, 2000 as 6,063,772, which is a continuation of US Ser. No. 08/590449 filed Jan 23, 1996, issued on June 16, 1998 as US 5,767,097.

### **FIELD OF THE INVENTION**

The field of the invention is immunology.

### **BACKGROUND OF THE INVENTION**

From seminal work by Mossman and Coffman (Mossmann TR, Coffmann RL: Th1 and Th2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* 1989, 7: 145-173), growth factors known as cytokines produced by T helper or CD4<sup>+</sup> T cells in both human and murine systems were classified into two subsets, Th1 and Th2. These were characterized by their functions in regulating various types of immune responses. Cytokines produced by Th1 cells [interleukin (IL)-2, interferon-alpha (IFN $\gamma$ ), tumor necrosis factor-alpha (TNF $\alpha$ ), IL-12] stimulated strong cellular immunity whereas Th2 cytokines [IL-4, IL-5, IL-6, IL-10, IL-13] were important for eliciting humoral (antibody) responses *in vivo*. Recently cytokines produced by non-CD4<sup>+</sup> T cells have been shown to be important in *in vivo* responses. In particular, the cytotoxic or CD8<sup>+</sup> T cells can also be subdivided into two subgroups, Tc1 and Tc2, which correspond to the same subsets in T helper cells (Carter LL, Dutton RW: Type 1 and Type 2: a functional dichotomy for all T cell subsets. *Curr Opin Immunol* 1996, 8: 336-342). This has led to the current nomenclature being generalized from Th1/Th2 to Type 1/Type 2 to reflect more closely the response generated by particular cytokines, rather than the cell types that produces them.

At the time the original application was filed for the recently issued patent (Specific modulation of Th1/Th2 cytokine expression by ribavirin in activated T cells - R. Tam, #5,767,097), the nomenclature of Type 1 and Type 2 had not been universally adopted. We thus used the Th1/Th2 nomenclature prevalent at the time of the original filing to include both CD4<sup>+</sup>

and CD8<sup>+</sup> T cells, as shown in the 'Background' section of that application (column 1, line 14). In this application we employ the terms, Type 1 and Type 2, instead of the previously used terms, Th1/Th2.

Strongly polarized Type 1 and Type 2 responses not only play different roles in protection, they can promote different immunopathological reactions. Type 1-type responses are involved organ specific autoimmunity such as experimental autoimmune uveoretinitis (Dubey et al, 1991, *Eur Cytokine Network* **2** : 147-152), experimental autoimmune encephalitis (EAE) (Beraud et al, 1991, *Cell Immunol* **133** : 379-389) and insulin dependent diabetes mellitus (Hahn et al, 1987, *Eur J Immunol* **18** : 2037-2042), in contact dermatitis (Kapsenberg et al, *Immunol Today* **12** : 392-395), and in some chronic inflammatory disorders. In contrast Type 2-type responses are responsible for triggering allergic atopic disorders (against common environmental allergens) such as allergic asthma (Walker et al, 1992, *Am Rev Resp Dis* **148** : 109-115) and atopic dermatitis (van der Heijden et al, 1991, *J Invest Derm* **97** : 389-394), are thought to exacerbate infection with tissue-dwelling protozoa such as helminths (Finkelman et al, 1991, *Immunoparasitol Today* **12** : A62-66) and *Leishmania major* (Caceres-Dittmar et al, 1993, *Clin Exp Immunol* **91**: 500-505), are preferentially induced in certain primary immunodeficiencies such as hyper-IgE syndrome (Del Prete et al, 1989, *J Clin Invest* **84**: 1830-1835) and Omenn's syndrome (Schandene et al, 1993, *Eur J Immunol* **23**: 56-60), and are associated with reduced ability to suppress HIV replication (Barker et al, 1995, *Proc Soc Nat Acad Sci USA* **92**: 11135-11139).

Thus, it is clear that modulation of the lymphokine profiles of the aforementioned disease states would be of therapeutic benefit. Promoting a Type 1 response would most likely lead to the reversal of a Type 2 phenotype and vice versa. Monoclonal antibodies (mAb) to lymphokines, lymphokines themselves and other agents such as thiol antioxidants (Jeannin et al, 1995, *J Exp Med* **182**: 1785 - 1792) have been shown to reverse the pathogenesis of certain diseases by inhibiting the disease-promoting cytokine pattern, either Type 1 or Type 2. For example, intracellular protozoan infections are limited by IFN $\gamma$  but exacerbated by IL-4, while nematode infections are controlled by IL-4 and exacerbated by IFN $\alpha$  (Heinzel et al, 1989, *J Exp Med* **162**:

59-72, Else et al, 1994, *J Exp Med* **179**: 347-351). Insulin-dependent diabetes mellitus in NOD mice and EAE in mice and rats can be ameliorated by treatment with IL-4 or anti- IFN $\gamma$  mAb before development of the disease (Rapoport et al, 1993, *J Exp Med* **178**: 87-99, Racke et al, 1994, *J Exp Med* **180**: 1961-1966, Campbell et al, 1991, *J Clin Invest* **87**: 739-742). In addition, autoimmune graft versus host disease (GVHD) that is characterized by a systemic lupus erythematosus-like syndrome is associated with Type 2 lymphokine production and is inhibited by anti-IL-4 antibody (Umland et al, 1992, *Clin Immunol Immunopathol* **63**: 66-73). On the other hand, Type 1 cytokines are produced in acute GVHD, in which donor CD8<sup>+</sup> T cells develop into CTL and destroy the host immune system. Treatment with anti-IFN $\gamma$  or anti-TNF $\alpha$  mAb ameliorates disease, and treatment with anti-IL-2 mAb converts acute GVHD to autoimmune GVHD (Via and Finkelman, 1993, *Int Immunol* **5**: 565-572).

Clinical trials of native and recombinant IL-2 in treating HIV-infected patients have been in progress since 1983 (Volberding et al, 1987, *AIDS Res Hum Retroviruses*, **3**: 115-124). Here, the relationship comes from the fact that development of AIDS has been reported to be associated with a shift in the pattern of lymphokines produced (Clerici and Shearer, 1994, *Immunol Today* **15**: 575-581). Over time, in an infected individual progressing towards disease, a decreased expression of Type 1 lymphokines such as IL-2 occurs (Maggi et al, 1987, *Eur J Immunol* **17**: 1685-1690, Gruters et al, 1990, *Eur J Immunol* **20**: 1039-1044, Clerici et al, 1993, *J Clin Invest* **91**: 759-765), concomitant with an increased production of Type 2 lymphokines such as IL-4 and IL-10 (Clerici et al, 1994, *J Clin Invest* **93**: 768-775, Hoffman et al, 1985, *Virology* **147**: 326-335). T-cells from asymptomatic or long term survivors treated with IL-2 enhanced their anti-HIV activity whereas exposure to IL-4 or IL-10 reduced their ability to suppress HIV replication and to produce IL-2 (Barker et al, 1995, *Proc Soc Nat Acad Sci USA* **92**: 11135-11139).

These current immunomodulatory therapeutics (mAbs and recombinant cytokines) are, however, not without limitations. For example with chronic monoclonal antibody treatment, the host animal develops antibodies against the monoclonal antibodies thereby limiting their usefulness. 'Humanized' monoclonal antibodies have been developed which apparently reduces

the risk of an induced immune response to these mAbs. However, these are still under development, and in addition these new mAbs remain large proteins and therefore may have difficulty reaching their target sites. Cytokine-based therapeutics also have limitations. For example, IL-12 treatment of autoimmune GVHD leads to the development of acute GVHD in mice.

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is a synthetic nucleoside capable of inhibiting RNA and DNA virus replication (Huffman et al, 1973, *Antimicrob. Agents Chemother* 3: 235, Sidwell et al, 1972, *Science* 177: 705). We have confirmed the observations of others who suggested that Ribavirin, in addition to its antiviral activity, has an effect on certain immune responses (reviewed Jolley and Suchil, 1984, Clinical Applications of Ribavirin: p93-96). We have also confirmed observations of others that Ribavirin affects the proliferation of mitogen- and antigen-activated T and B lymphocytes, (Tam et al, 1995 (data not shown), Peavy et al, 1980, *Infection and Immunity* 29: 583-589) and then when combined with cyclosporin, Ribavirin showed efficacy in long term allograft survival, Jolley et al (1988, *Transplantation Proc* 20: 703-706).

In addition, we have significantly advanced the prior research by demonstrating that Ribavirin modulates the cytokine pattern of an immune response at least in part by promoting a Type 1 response and suppressing a Type 2 response. In hindsight, this discovery is not inconsistent with prior research. First, Ribavirin is known to inhibit both functional humoral immune responses, (Peavy et al, 1981, *J Immunol* 126: 861-864, Powers et al, 1982, *Antimicrob Agents Chemother* 22: 108-114) and IgE-mediated modulation of mast cell secretion (Marquardt et al, 1987, *J Pharmacol Exp Therapeutics* 240: 145-149, (both Type 2 lymphokine-mediated events). Second, Ribavirin antagonizes the antiviral effect of azidothymidine (AZT) in peripheral blood lymphocytes from HIV patients (Vogt et al, 1987, *Science* 235: 1376-1379). This finding is significant because AZT decreases IL-2 receptor (IL-2R) but not IL-2 expression (Viora and Camponeschi, 1995, *Cell Immunol* 163: 289-295). It is therefore possible that Ribavirin antagonizes AZT by modulating IL-2 expression and elevating depressed levels of IL-2R. Third, Ribavirin treatment of an immunocompromised patient for chronic GVHD (a Type 2-mediated

disorder) led to a dramatic resolution of the disease, an outcome which did not occur with conventional immunosuppressive therapies such as cyclosporin and glucocorticoids (Cassano, 1991, *Bone Marrow Transplantation* **7**: 247-248). Finally, Ribavirin treatment (one year) of patients for hepatitis C (HCV) revealed fewer lymphocyte aggregates and far less liver damage than placebo controls (Dusheiko et al, 1994, *Hepatology* **20**: 206A). This observation may reflect the fact that although, the predominant immune response to hepatitis C is mediated by Type 1 lymphokines, T cells of the Type 2 phenotype can be infected by HCV (Zignego et al, 1994, unpublished data) and this infection may drive further antibody-mediated destruction of hepatocytes.

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

FIGURE 1A is a graphical representation of the effect of Ribavirin and interferon alpha on the extracellular expression of IL-2 in PMA/ionomycin-activated T lymphocytes. Results are expressed as percentage of the increased lymphokine expression following PMA/ionomycin treatment alone.

FIGURE 1B is a graphical representation of the effect of Ribavirin and interferon alpha on the extracellular expression of TNF $\alpha$  in PMA/ionomycin-activated T lymphocytes. Results are expressed as percentage of the increased lymphokine expression following PMA/ionomycin treatment alone.

FIGURE 1C is a graphical representation of the effect of Ribavirin and interferon alpha on the extracellular expression of IL-4 in PMA/ionomycin-activated T lymphocytes. Results are expressed as percentage of the increased lymphokine expression following PMA/ionomycin treatment alone.

FIGURE 1D is a graphical representation of the effect of Ribavirin and interferon alpha on the extracellular expression of IFN $\gamma$  in PMA/ionomycin-activated T lymphocytes. Results are expressed as percentage of the increased lymphokine expression following PMA/ionomycin treatment alone.

FIGURE 2A is a graphical representation of the effect of 2, 10 or 50  $\mu$ M Ribavirin in the presence of 2000 U/ml interferon alpha (left panels) and the effect of 500, 1000 or 2000 U/ml interferon alpha (right panels) in the presence of 10  $\mu$ M Ribavirin on the extracellular expression of IL-2 in PMA/ionomycin-activated T lymphocytes.

5        FIGURE 2B is a graphical representation of the effect of 2, 10 or 50  $\mu$ M Ribavirin in the presence of 2000 U/ml interferon alpha (left panels) and the effect of 500, 1000 or 2000 U/ml interferon alpha (right panels) in the presence of 10  $\mu$ M Ribavirin on the extracellular expression of IL-4 in PMA/ionomycin-activated T lymphocytes.

FIGURE 2C is a graphical representation of the effect of 2, 10 or 50  $\mu$ M Ribavirin in the presence of 2000 U/ml interferon alpha (left panels) and the effect of 500, 1000 or 2000 U/ml interferon alpha (right panels) in the presence of 10  $\mu$ M Ribavirin on the extracellular expression of IL-2 in PMA/ionomycin-activated T lymphocytes.

FIGURE 2D is a graphical representation of the effect of 2, 10 or 50  $\mu$ M Ribavirin in the presence of 2000 U/ml interferon alpha (left panels) and the effect of 500, 1000 or 2000 U/ml interferon alpha (right panels) in the presence of 10  $\mu$ M Ribavirin on the extracellular expression of IL-4 in PMA/ionomycin-activated T lymphocytes.

FIGURE 3 is a graphical representation of the effect of Ribavirin and interferon alpha on IL-2, IL-4 and IFN $\gamma$  mRNA expression in PMA/ionomycin-activated T lymphocytes.

FIGURE 4A is a graphical representation of the effect of Ribavirin and interferon alpha on the cell surface expression of IL-2 receptors in PMA/ionomycin-activated T lymphocytes. Results are expressed as percentage of the increased lymphokine receptor expression following PMA/ionomycin treatment alone.

FIGURE 4B is a graphical representation of the effect of Ribavirin and interferon alpha on the cell surface expression of IL-4 receptors in PMA/ionomycin-activated T lymphocytes. Results are expressed as percentage of the increased lymphokine receptor expression following PMA/ionomycin treatment alone.

FIGURE 5A is a graphical representation of the expression of intracellular IL-2 expression in resting CD4<sup>+</sup> T cells. Data from one experiment is shown and represented as the percentage of cells showing double positive staining for IL-2 and CD4 or CD8.

FIGURE 5B is a graphical representation of the expression of intracellular IL-2 expression in activated CD4<sup>+</sup> T cells treated with PMA/ionomycin alone. Data from one experiment is shown and represented as the percentage of cells showing double positive staining for IL-2 and CD4 or CD8.

FIGURE 5C is a graphical representation of the expression of intracellular IL-2 expression in activated CD4<sup>+</sup> T cells in the presence of 10  $\mu$ M Ribavirin. Data from one experiment is shown and represented as the percentage of cells showing double positive staining for IL-2 and CD4 or CD8.

FIGURE 5D is a graphical representation of the expression of intracellular IL-2 expression in activated CD4<sup>+</sup> T cells treated with 5000 U/ml interferon alpha. Data from one experiment is shown and represented as the percentage of cells showing double positive staining for IL-2 and CD4 or CD8.

FIGURE 5E is a graphical representation of the expression of intracellular IL-2 expression in resting CD8<sup>+</sup> T cells. Data from one experiment is shown and represented as the percentage of cells showing double positive staining for IL-2 and CD4 or CD8.

FIGURE 5F is a graphical representation of the expression of intracellular IL-2 expression in activated CD8<sup>+</sup> T cells treated with PMA/ionomycin alone. Data from one experiment is shown and represented as the percentage of cells showing double positive staining for IL-2 and CD4 or CD8.

FIGURE 5G is a graphical representation of the expression of intracellular IL-2 expression in activated CD8<sup>+</sup> T cells in the presence of 10  $\mu$ M Ribavirin. Data from one experiment is shown and represented as the percentage of cells showing double positive staining for IL-2 and CD4 or CD8.

FIGURE 5H is a graphical representation of the expression of intracellular IL-2 expression in activated CD8<sup>+</sup> T cells treated with 5000 U/ml interferon alpha. Data from one experiment is shown and represented as the percentage of cells showing double positive staining for IL-2 and CD4 or CD8.

5        FIGURE 6A is a graphical representation of a contemplated Ribavirin analog.

FIGURE 6B is a graphical representation of a contemplated Ribavirin analog.

FIGURE 6C is a graphical representation of a contemplated Ribavirin analog.

FIGURE 6D is a graphical representation of a contemplated Ribavirin analog.

FIGURE 7A is a graph showing the results of various concentrations of Ribavirin analogs on IL-2.

FIGURE 7B is a graph showing the results of various concentrations of Ribavirin analogs on TNF- $\alpha$ .

FIGURE 7C is a graph showing the results of various concentrations of Ribavirin analogs on IFN- $\gamma$ .

FIGURE 7D is a graph showing the results of various concentrations of Ribavirin analogs on IL-4.

FIGURE 7E is a graph showing the results of various concentrations of Ribavirin analogs on IL-5.

#### **SUMMARY OF THE INVENTION**

20        In accordance with the present invention, the nucleoside, Ribavirin, is administered to a patient in a dosage range which is effective to modulate lymphokine expression in activated T cells. In particular, Ribavirin is used to suppress Type 2-mediated T cell responses and promote Type 1-mediated T cell response.

25        Thus, instead of administering Ribavirin in its well-recognized role as an anti-viral agent, Ribavirin is herein used in the treatment of imbalances in lymphokine expression. Such



[illegible]

5 Ribavirin is preferably administered orally to a human patient in a dosage which achieves  
a blood serum level averaging about 0.25 to about 6.7  $\mu\text{M}$ .”

Table 1		
Mg/day	mg/kg/day	$\mu$ M
800	11.4	6.7
600	8.6	5.0
400	5.7	3.3
300	4.3	2.5
200	2.9	1.7
125	1.8	1.0
60	0.9	0.50
30	0.4	0.25

Table 2, for comparison, gives the previously known dosage ranges.

Table 2				
mg/day	mg/kg/day	$\mu$ M		
1500	21.4	12.5		highest level of oral administration
1200	17.1	10.0		level at which anemia is problematic
1000	14.3	8.3		lowest level of prior art antiviral use

Since Ribavirin has been on the market for several years, many dosage forms and routes of administration are known, and all appropriate dosage forms and routes of administration may be utilized. For example, in addition to oral administration, Ribavirin may given intravenously, intramuscularly, intraperitoneally, topically, and the like, all of which are known. Pharmaceutical formulations comprising Ribavirin may also comprise one or more pharmaceutically acceptable carriers, which may include excipients such as stabilizers (to promote long term storage), emulsifiers, binding agents, thickening agents, salts, preservatives, solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the Ribavirin, its use in

the therapeutic compositions and preparations is contemplated. Supplementary active ingredients can also be incorporated into the compositions and preparations.

It is especially contemplated that Ribavirin may be administered, in the doages set forth or interpolated from Table 1. Such administration may occur as a monotherapy, or in  
5 conjunction with another antiviral agent such as interferon alpha.

In addition to the therapeutic uses of Ribavirin contemplated herein, Ribavirin may also be used as a laboratory tool for the study of absorption, distribution, cellular uptake, and efficacy.

Still further, it is contemplated that Ribavirin may be used to treat or otherwise mitigate the effects of drug induced impairment of immune function. A drug of particular interest in this regard is ethanol, ordinary drinking alcohol. Long term, excessive ingestion of ethanol has recently been shown to favor Type 2 rather than Type 1 cytokines. Zisman, D. A., et al, "Ethanol feeding impairs innate immunity and alters the expression of Th1 and Th2-phenotype cytokines in murine Klebsiella pneumonia", Alcohol Clin Exp Res, vol. 22, no. 3, 1998 May, pp 621-7. Other drugs are known or suspected of having similar effects.

## EXAMPLES

### Cell Lines And T Cell Purification

Peripheral blood mononuclear cells (PBMCs) were isolated from the buffy coat following Ficoll-Hypaque density gradient centrifugation of 60 ml blood from healthy donors. T-cells  
20 were then purified from the PBMCs using Lymphokwik lymphocyte isolation reagent specific for T-cells (LK-25T, One Lambda, Canoga Park CA). An average yield of 40 - 60 x 10<sup>6</sup> T-cells were then incubated overnight at 37 °C in 20 - 30 ml RPMI-AP5 (RPMI-1640 medium (ICN, Costa Mesa, CA) containing 20 mM HEPES buffer, pH 7.4, 5 % autologous plasma, 1 % L-glutamine, 1 % penicillin/streptomycin and 0.05 % 2-mercaptoethanol) to remove any  
25 contaminating adherent cells. In all experiments, T-cells were washed with RPMI-AP5 and then plated on 96-well microtitre plates at a cell concentration of 1 x 10<sup>6</sup> cells/ml.

### **T-Cell Activation And Ribavirin Treatment**

T-cells were activated by the addition of 500 ng ionomycin and 10 ng phorbol 12-myristate 13-acetate (PMA) (Calbiochem, La Jolla, CA) and incubated for 48 - 72h at 37 °C. PMA/ionomycin-activated T-cells were treated with 0.5 - 50 µM Ribavirin or with 250 - 10000 U/ml of a control antiviral, interferon-alpha (Accurate, Westbury, NY) immediately following activation and re-treated 24 h later. T-cells from each plate were used for immunofluorescence analysis and the supernatants used for extracellular cytokine measurements. Following activation, 900 µl cell supernatant from each microplate was transferred to another microplate for analysis of cell-derived cytokine production. The cells are then used in immunofluorescence analyses for intracellular cytokine levels and cytokine receptor expression.

### **Extracellular Cytokine Analyses**

Cell-derived human cytokine concentrations were determined in cell supernatants from each microplate. Activation-induced changes in interleukin-2 (IL-2) levels were determined using a commercially available ELISA kit (R & D systems Quantikine kit, Minneapolis, MN) or by bioassay using the IL-2-dependent cell line, CTLL-2 (ATCC, Rockville, MD). Activation - induced changes in interleukin-4 (IL-4), tumor necrosis factor (TNFα) interleukin-8 (IL-8) (R & D systems (Quantikine kit, Minneapolis, MN) and interferon-gamma (IFN-α) (Endogen (Cambridge, MA) levels were determined using ELISA kits. All ELISA results were expressed as pg/ml and the CTLL-2 bioassay as counts per minute representing the IL-2-dependent cellular incorporation of <sup>3</sup>H-thymidine (ICN, Costa Mesa, CA) by CTLL-2 cells.

### **Direct Immunofluorescence Studies (Cytokine Receptors)**

For direct staining with fluorescence-conjugated antibodies to cell surface antigens, the cells were washed twice with isotonic saline solution, pH 7.4 (Becton Dickinson, Mansfield, MA) and resuspended in 50 µl isotonic saline solution and split into two samples. One sample aliquot was co-stained with either PE-anti CD25/FITC-anti CD4 or PE-rat anti mouse IgG + anti CDw124/FITC-anti CD4 mAb and non-specific fluorescence was assessed by staining the second aliquot with PE/FITC-labeled isotype-matched control monoclonal antibody. All fluorescence-

labeled monoclonal antibodies were obtained from Becton Dickinson (San Jose, CA) except for anti-CDw124 which was obtained from Pharmingen, San Diego, CA. Incubations were performed at 4 °C in the dark for 45 min using saturating mAb concentrations. Unincorporated label was removed by washing in PBS prior to the analysis with a FACScan flow cytometer (Becton Dickinson).

Antigen density was indirectly determined in gated live CD4<sup>+</sup> T cells and expressed as the mean channel of fluorescence (MCF). Surface expression of specific antigen (CDw124, CD25) was represented as the mean channel shift (MCS) obtained by subtracting the MCF of FITC- or PE-labeled isotype-matched (IgG1) control mAb-stained cells from the MCF of FITC- or PE-labeled antigen-specific mAb stained cells. Alternatively, surface expression of the CD4<sup>+</sup>-subset of cells stained with CD28 mAb was determined by subtracting the MCF of CD28<sup>+</sup> CD4<sup>+</sup> from the MCF of CD28<sup>-</sup> CD4<sup>-</sup> cells.

The viability of control untreated and Ribavirin and interferon  $\alpha$ -treated cells were determined in each batch of all oligonucleotides in multiple donors by staining with the vital dye, propidium iodide (5  $\mu$ g/ml final concentration). The percentage of live cells which excluded propidium iodide was determined by flow cytometry and was > 90 % (range 90 - 99 %) following treatment with all concentrations used.

#### **Immunofluorescence Analyses of Intracellular Cytokine Expression**

For analyses of the intracellular expression of IL-2 in CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets, T cells were first treated for the last 4 h of 48 - 72h activation with 10  $\mu$ g Brefeldin A (Gibco BRL, Gaithersburg, MD) to minimize secretion of newly synthesized IL-2 into the extracellular milieu. Following activation, 900  $\mu$ l cell supernatant from each microplate was transferred to another microplate for analysis of cell-derived cytokine production. Prior to direct staining (30 min, 4 °C, in the dark) with FITC-conjugated antibodies to the cell surface antigens, CD4 and CD8, the cells were washed twice with isotonic saline solution, pH 7.4 and resuspended in 100 - 150  $\mu$ l Staining Buffer (phosphate buffered saline, pH 7.4 containing 1% Fetal Calf Serum (FCS) (Hyclone,

Logan, UT) and 0.1% sodium azide), and split into two samples. Stained cells were washed in 1 ml Staining Buffer and cell pellet resuspended in 100 µl Fixation Buffer (4% paraformaldehyde in PBS) following aspiration of the supernatant. Fixed cells were kept at 4 C for 20 mins, then washed in 1 ml Staining Buffer and cell pellet resuspended with mixing in 50 µl

- 5 Permeabilization Buffer (0.1% saponin (ICN, Costa Mesa, CA) in PBS). Permeabilized cells were stained with PE-labeled IL-2 antibody for 30 min at 4 C in the dark and then washed in 1 ml Permeabilization Buffer, resuspended in 250 µl Staining Buffer prior to FACS analysis.

#### **Analysis of Cytokine mRNA**

Total RNA was extracted from resting T cells and from Ribavirin and interferon  $\alpha$ -treated and untreated activated T cells using a commercial variation of the guanidium thiocyanate/phenol extraction technique (Trizol reagent (GIBCO/BRL). RNA was washed with 70% ethanol and finally resuspended in 10 µl DEPC-treated water.

cDNA synthesis reaction was performed as per manufacturers instructions (Promega, Madison, WI). Briefly, 1 µg of total RNA was heated at 65<sup>0</sup> C for 10 min and cooled on ice before combining with 2 µl 10X reverse transcription buffer (100 mM Tris HCl (pH 8.8), 500 mM KCl, 1% Triton X-100), 5 mM MgCl, 2 µl 10 mM dNTPs (1 mM each dNTP), 0.5 µl RNase inhibitor, 1 µl oligo (dT)<sub>15</sub> primer (0.5 µg/ µg RNA) and 0.65 µl AMV reverse transcriptase (H. C.). The reaction was incubated at 42<sup>0</sup>C for 1 h followed by at 95<sup>0</sup>C for 10 min and 5 min on ice.

- 20 The PCR reaction was performed using GeneAmp PCR kit (Perkin-Elmer Cetus, Foster City, CA). In a fresh tube, RT reaction mixture (3 µl) was combined with 5 µl 10 X PCR buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.3, 15 mM MgCl<sub>2</sub> and 0.01% (w/v) gelatin), 1 µl 10 mM dNTPs and 1 U of Taq DNA polymerase. The primers used were as follows: interleukin-2, interleukin-4, interferon- $\gamma$  (human) primers (Stratagene, La Jolla, CA) and pHE7 ribosomal gene. Amplification conditions were 45 sec at 94<sup>0</sup>C, 1 min at 57<sup>0</sup>C and 2 min at 72<sup>0</sup>C for 35 cycles, 25 followed by 8 min at 72<sup>0</sup>C. PCR products were analyzed on 2% agarose gel containing ethidium bromide. Following electrophoresis, PCR products were transferred to Hybond N+ membrane (Amersham, Arlington Heights, IL) in 20 X SSC overnight and immobilized using 0.4 M NaOH.

Blots were hybridized with  $^{32}\text{P}$ - $\gamma$ ATP labeled oligonucleotide probes in Rapid - hyb buffer (Amersham) for 1 h at  $42^{\circ}\text{C}$ . Each cytokine primer mix was used as a radiolabeled probe (as per instructions). Equivalent loading was assessed following hybridization with a probe generated from pHE7 sense primer. Washed blots were then analyzed using PhosphorImager.

#### **Effect of Ribavirin on Extracellular Cytokine Levels in Activated T Cells**

PMA/ionomycin treatment (48 - 72h) of human T-cells substantially increased the levels of all the cytokines analyzed i.e. IL-2, IL-4,  $\text{TNF}\alpha$ ,  $\text{IFN}\gamma$  (Table 1). The first number in each cell depicts the arithmetic mean, and the numbers in parenthesis depicts the relevant ranges.  $N = 4$ . In a representative experiment shown in Figure 1, addition of Ribavirin, in the dose range 0.5 - 50  $\mu\text{M}$ , augmented activated levels of the Type 1 cytokines, IL-2 and  $\text{TNF}\alpha$  maximally at 5  $\mu\text{M}$  (30%) and 20  $\mu\text{M}$  (36%) respectively. In contrast, interferon- $\alpha$ , inhibited IL-2 and  $\text{TNF}\alpha$  expression in a dose-dependent manner (range 250 - 10000 U/ml, maximal inhibition 33 and 38% respectively), when compared to levels in untreated activated T cells. In addition, Ribavirin mediated a concomitant decrease in activated levels of the Type 2 cytokine, IL-4 (peak inhibition of 74% at 2  $\mu\text{M}$ ) whereas interferon- $\alpha$  maximally increased extracellular IL-4 by 26% (10000 U/ml). Using combinations of Ribavirin and interferon alpha, Figure 2 shows that a constant 2000 U/ml of interferon alpha suppressed the Ribavirin dose-dependent augmentation of activated IL-2 levels (A) and reversed the inhibition of activated IL-4 levels (C). Similarly, a constant 10  $\mu\text{M}$  of Ribavirin reversed the interferon alpha-mediated dose-dependent inhibition of activated IL-2 levels (B) and suppressed the augmentation of activated IL-4 levels (D).

#### **Effect of Ribavirin on Cytokine mRNA Levels in Activated T Cells**

These opposing effects of Ribavirin and interferon- $\alpha$  on activated extracellular cytokine levels were also observed at the level of transcription. Figure 3 shows that PMA/ionomycin treatment of human T-cells substantially augments IL-2, IL-4 and  $\text{IFN}\gamma$  mRNA levels. Treatment with Ribavirin (2, 5 and 10  $\mu\text{M}$ ) following T cell activation, elevates IL-2, decreases IL-4 and has no effect on  $\text{IFN}\gamma$  mRNA. In contrast, interferon  $\alpha$ , at 1000, 2000 and 5000 U/ml decreases IL-2, increases IL-4 and decreases  $\text{IFN}\gamma$  mRNA. Therefore the respective dose-dependent effects of

Ribavirin and interferon  $\alpha$  on IL-2, TNF $\alpha$ , and IL-4 mRNA expression paralleled the ELISA analyses. These data suggest that Ribavirin promotes the synthesis of the Type 1 cytokines, IL-2 and TNF $\alpha$  and inhibits the expression of the Type 2 cytokine, IL-4 in activated human T cells.

#### **Effect of Ribavirin on IL-2 and IL-4 Receptor Levels in Activated T Cells**

5 Using FACS analysis, we compared the effects of Ribavirin and interferon  $\alpha$  on expression of IL-2 (CD25) and IL-4 (CDw124) receptor expression in activated T cells. PMA/ionomycin-treatment increases CD25 and CDw124 expression from resting levels of  $50.16 \pm 0.45$  and  $62.31 \pm 1.46$  to activated levels of  $162.48 \pm 2.89$  and  $87.53 \pm 3.98$  respectively (n = 4). In a representative of 3 experiments, Figure 4 shows that Ribavirin (1 - 50  $\mu$ M) has little effect on activated levels of IL-2 and IL-4 receptor whereas interferon  $\alpha$  in the dose range 250 - 10000 U/ml, decreased IL-2 receptor and increased IL-4 receptor expression in a dose-dependent manner, when compared to receptor levels in control activated T cells. Therefore, these data show that the effect of Ribavirin on cytokine synthesis acts independently of cytokine receptor expression. In contrast, the effect of interferon  $\alpha$  treatment on IL-2 and IL-4 receptor correlates with that observed with its effect on activated IL-2 and IL-4 expression.

#### **Effect of Ribavirin on Intracellular IL-2 Levels in CD4 and CD8<sup>+</sup> Subsets of Activated T Cells**

We examined whether the effect of Ribavirin on IL-2 expression was specific to CD4<sup>+</sup> or CD8<sup>+</sup> T cells. Intracellular IL-2 expression in fixed and Permeabilized activated T cells was determined by two-color flow cytometry using fluorescence-labeled antibodies to CD4 or CD8 and to IL-2. Figure 5 shows that following treatment with Ribavirin at 10  $\mu$ M, the percentage of CD4<sup>+</sup> T cells expressing IL-2 rose from 82 to 91% and the percentage of CD8<sup>+</sup> expressing IL-2 increased from 81 to 91%. In contrast, the percentage of IL-2-expressing CD4<sup>+</sup> and CD8<sup>+</sup> cells following interferon  $\alpha$  treatment (5000 U/ml) was 81 and 71% respectively. These data suggest Ribavirin has an effect on intracellular IL-2 expression which does not discriminate between CD4<sup>+</sup> or CD8<sup>+</sup> T cell subsets. In contrast, interferon  $\alpha$  treatment has little effect on CD4<sup>+</sup> T cells and even reduces IL-2 expression in the CD8<sup>+</sup> T cell subset.



[illegible]